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The structure of ends determines the pathway choice and Mre11 nuclease dependency of DNA double-strand break repair

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ABSTRACT

The key event in the choice of repair pathways for DNA double-strand breaks (DSBs) is the initial processing of ends. Non-homologous end joining (NHEJ) involves limited processing, but homology-dependent repair (HDR) requires extensive resection of the 5′ strand. How cells decide if an end is channeled to resection or NHEJ is not well understood. We hypothesize that the structure of ends is a major determinant and tested this hypothesis with model DNA substrates in *Xenopus* egg extracts. While ends with normal nucleotides are efficiently channeled to NHEJ, ends with damaged nucleotides or bulky adducts are channeled to resection. Resection is dependent on Mre11, but its nuclease activity is critical only for ends with 5′ bulky adducts. CtIP is absolutely required for activating the nuclease-dependent mechanism of Mre11 but not the nuclease-independent mechanism. Together, these findings suggest that the structure of ends is a major determinant for the pathway choice of DSB repair and the Mre11 nuclease dependency of resection.

INTRODUCTION

DNA double-strand breaks (DSBs) are among the most dangerous damages to the genome. They can arise from exposure to a plethora of DNA damaging agents such as ionizing radiation, anti-cancer drugs, and cellular metabolites (1–3). They are also intentionally introduced to execute specific biological processes such as Spo11-induced meiotic cross-over, RAG-mediated V(D)J recombination, AID-induced class switch recombination, and topoisomerase 2-catalyzed changes in DNA topology (4–6). Proper repair of DSBs is essential to the stability of the genome and failure to do so underlies many human diseases like immunodeficiency, premature aging, and most importantly, cancer (1,2,7,8). Clinically, some of the mainstay cancer treatments, such as the topoisomerase inhibitors etoposide and camptothecin, act by inducing DSBs in cells (6,9,10). Elucidating the mechanism for DSB repair is thus of fundamental importance to the understanding of genome instability in cancer development and holds great potential in improving anti-cancer treatment (10–13).

There are two relatively well studied DSB repair pathways: non-homologous end joining (NHEJ), and homology-dependent repair (HDR) (1,14,15). NHEJ is orchestrated by the end binding protein Ku and accomplished by directly ligating the ends, usually after minor processing. HDR consists of two subtypes: homologous recombination (HR) and single-strand annealing (SSA). HR repairs DSBs by invading a homologous sequence to copy the missing information. If a DSB occurs between two direct repeat sequences, it can also be repaired by SSA and the final product retains effectively only one of the two repeats. The key event in the bifurcation of NHEJ and HDR is the initial processing of DNA ends (16,17). NHEJ involves no or limited processing of ends, HDR requires resection of 5′ strands to form 3′ ss-tails. The choice of DSB repair pathways is thus dictated by the factors that determine if a DNA end is channeled to resection. One critical regulatory factor is cell cycle stage, as resection and HDR are active only during S and G2 phases (16). However, NHEJ and HDR are both active during S and G2, so additional factors must exist that affect DSB repair pathway choice. One potential factor is the structure of DNA ends. While ends with normal nucleotides are efficiently repaired by NHEJ, most clinically relevant ends, such as those generated by ionizing radiation or cancer drugs, have damaged nucleotides or bulky adducts (1,3). Such damaged ends are difficult to repair by NHEJ and thus pose a particularly serious problem to the genome during replication (8,18). It is reasonable to hypothesize that S phase cells might channel damaged ends to resection rather than wait for NHEJ.

DSB resection is carried out mainly by two pathways, one catalyzed by the 5′→3′ ds-DNA exonuclease Exo1 and the other by the combined actions of a RecQ-type DNA helicase, the 5′→3′ ss-DNA exonuclease DNA2, and the ss-DNA binding protein replication protein A (RPA) (19–24). The initiation of resection usu-

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ally requires the Mre11/Rad50/Nbs1 complex (MRN) (Mre11/Rad50/Xrs2 (MRX) in budding yeast) and Sae2 (CtIP in higher eukaryotes), but the underlying mechanism is still not well understood. The current model emphasizes the central role of the nuclease activity of Mre11, which has both 3′→5′ DNA exonuclease activity and ss-DNA endonuclease activity (25–29). Largely based on the study of DSBs with 5′-linked Spo11 in meiotic yeast cells, it has been proposed that the ss-DNA endonuclease activity of Mre11 makes an initial cut near the 5′ end and then the 3′→5′ exonuclease activity of Mre11 digests DNA in the 3′→5′ direction while Exo1 (and DNA2 in mitotic cells) degrade DNA in the 5′→3′ direction for extensive resection (30). This model is supported by many studies showing that the nuclease activity of Mre11 is important for resection of ionizing radiation induced DSBs (31–33). In particular, a recent study suggests that at ends with bulky adducts, Sae2 can activate a cryptic ds-DNA endonuclease activity in Mre11 to cleave the 5′ strand (34). However, it is also known that the Mre11 nuclease activity is not important for HO-endonuclease induced DSBs in mitotic yeast cells (35). Furthermore, in biochemical reconstitution experiments with purified proteins and clean ends, MRN/MRX is sufficient to stimulate resection even in the absence of Sae2/CtIP (36–39). Sae2 can stimulate Exo1-mediated resection, but this stimulation is independent of Mre11 nuclease activity (40). The apparently conflicting observations might be the result of different types of DSBs used in these studies.

The *in vivo* studies of how different types of DSBs are repaired are hampered by the technical difficulty in generating ends with various defined structures. While it is possible to introduce clean DSBs with site-specific endonucleases, it is currently impossible to introduce a pure type of damaged ends in cells. For example, ionizing radiation and DNA damaging drugs such as bleomycin and topoisomerase inhibitors generate a myriad of damages, including ends with protein adducts, SSBs with protein adducts, stalled replication forks, or reversed replication forks (1,3). Therefore, *in vivo* studies can only assay for the composite outcome of multiple repair pathways. For 'dirty ends', the assay for repair poses an additional problem. One is forced to use indirect assays such as the formation and disappearance of γH2Ax foci as a proxy for DSB repair (18,41). To overcome this difficulty, we have used biochemical reconstitution in *Xenopus* egg extracts to gain more precise mechanistic insights into the role of end structure on the choice of DSB repair pathways. By using model substrates carrying different types of end structures, we found that ends with normal nucleotides are efficiently repaired by NHEJ, but ends with damaged nucleotides or bulky adducts are more refractory to NHEJ and channeled to resection. We also found that MRN is required for the resection of all the damaged ends. However, the nuclease activity of Mre11 is critical only for the resection of DSBs with 5′ bulky adducts, suggesting MRN can initiate resection by both a nuclease-dependent and a nuclease-independent mechanism. CtIP is absolutely required for activating the nuclease-dependent mechanism of Mre11 but not the nuclease-independent mechanism. Together, these findings reveal important insights into the regulation of DSB repair pathway and the role of Mre11 nuclease activity in resection.

MATERIALS AND METHODS

Preparation of *Xenopus* egg extracts and immunodepletion of *Xenopus* Mre11 and CtIP

Membrane-free extracts were prepared from unfertilized interphase *Xenopus* eggs following the published protocol (42). Mre11 and CtIP were depleted as previously described (38).

Expression and purification of recombinant *Xenopus* MRN complex

The nuclease-dead mutant Mre11 (H130S/N) was prepared using the QuikChange site-directed mutagenesis kit (Agilent, CA). The wild-type and mutant MRN complexes were purified by conventional column chromatography as previously described (38).

DNA resection assays in *Xenopus* egg extracts

The DNA substrates carrying damaged 3′ ends were prepared by linearizing a 5.7kb plasmid (pBLP) with BamHI and then filling the end with dGTP, ^{32}P-dATP, TTP, and dCTP/ddCTP/biotin-14-dCTP (Invitrogen, CA). The DNA substrates carrying damaged 5′ ends were prepared by PCR with a 5.7-kb plasmid template (pBS-N), Pfu DNA polymerase (Promega, WI), and oligonucleotides carrying 5′-phosphotyrosine, biotin, or hydroxyl groups (Midland, TX) in the presence of ^{32}P-labeled dATP. The products were purified first by Qiagen's PCR purification columns and then by gel-filtration with Sepharose CL-2B beads (Sigma-Aldrich, MO, USA). The peak fractions were pooled and concentrated to 50 ng/μl. To prepare avidin-bound DNA, the 3′ or 5′ biotin DNA was pre-incubated at 20 ng/μl with 4μg/μl avidin (Neutravidin; Pierce/ThermoScientific, IL) on ice for 5 minutes. A typical resection assay contained 5μl depleted extracts, 0.5μl 10× ATP mix (20 mM ATP/200mM phosphocreatine/0.5mg/ml creatine kinase/50 mM DTT), 1–1.5 ng/μl DNA, and ELB buffer (10 mM HEPES (pH 7.5)/50 mM KCl/2.5 mM $MgCl_2$/250 mM sucrose/1 mM DTT) or protein (total volume = 7.5 μl). The reactions were incubated at 22°C and samples were taken at the indicated times and mixed with an equal volume of 2% SDS/25mM EDTA. At the end, samples were brought up to 10 μl with H_2O and treated with 1 μl proteinase K (10 mg/ml) at 22°C for 2 h. The resection products were separated by 1% TAE/agarose gel electrophoresis and the gels were dried and exposed to Phosphorimager (Fuji) or film.

Analysis of resection intermediates

DNA intermediates were isolated from extracts by first incubating with 3 volumes of ELB buffer supplemented with 25 mM EDTA and 1/2 volume of proteinase K (10 mg/ml in H_2O) at 22°C for 2 h and then purified with the PCR purification columns following the manufacturer's protocol (Qiagen, CA, USA). To detect the presence of 3′ biotin on 3′ ss-overhangs or resection intermediates, the DNA was pre-incubated with ELB buffer or avidin on ice for 5 min, and then treated with *Escherichia coli* ExoI (NEB, MA) at 22°C

for 60 min. To analyze the intermediates of the 5′ biotin-avidin DNA, DNA was treated with *E. coli* ExoI (0.2 u/μl, NEB, MA) or RecJ (0.3 u/μl; NEB, MA) at 22°C for 60 min. To detect the presence of 5′ biotin, DNA was pre-incubated with ELB buffer or avidin on ice for 5 min, and then treated with T7 Exo (0.6 unit/μl; NEB, MA) at 22°C for 60 min. Reactions were analyzed by 1% TAE-agarose gel electrophoresis and the gels were first stained with SYBR Gold (Invitrogen, CA, USA) and then dried for exposure to film.

Analysis of MRN's effect on Exo1's activity towards 3′ avidin DNA

To assay the effect of 3′ avidin on exonuclease activity, the ^{32}P-labeled 3′ biotin substrate was pre-incubated at 20 ng/μl with ELB or 4 μg/μl avidin on ice for 5 min. The substrates were then incubated with lambda exonuclease (0.025 unit/μl; NEB, MA) or purified recombinant *Xenopus* Exo1 (0.25ng/μl) at 22°C. To assay the effect of the MRN complexes on the nucleases, the reactions also contained recombinant wild-type or mutant MRN proteins at 16 ng/μl. Samples were taken at the indicated times, mixed with equal volume of 2% SDS/25 mM EDTA. After the final time point was taken, samples were treated with proteinase K at 1 mg/ml at 22°C for 2 h and then analyzed by 1% TAE-agarose gel electrophoresis. The gels were first stained with SYBR Gold (Invitrogen, CA, USA) and then dried for exposure to film.

Data quantitation and analysis

The amounts of substrates remaining or products produced were quantitated by Fuji ImageQuan (Fuji). The averages and standard deviations were calculated and plotted. Comparisons of means were conducted by one-tailed T-tests.

RESULTS

DNA with 3′ damaged nucleotides or bulky adducts is channeled to resection

To mimic damaged 3′ ends, linear DNA substrates (5.7 kb in length) were prepared to carry a normal nucleotide (dC), a dideoxynucleotide (ddC), or a biotin nucleotide (biotin attached to the N4 position of dC via a 14-atom linker) at the 3′ end. The ddC mimicked damaged nucleotides and binding of the avidin protein (mw = 60 kDa) to biotin dC mimicked DNA with bulky adducts. The substrates also carried a ^{32}P-labeled nucleotide adjacent to the 3′ end to allow easy detection of DNA. The substrates were incubated with *Xenopus* egg extracts and the products taken at various times were analyzed by TAE-agarose gel electrophoresis. DNA with normal 3′ nucleotides was efficiently repaired into supercoiled and relaxed monomers, dimers, and multimers (Figure 1A&B). This type of repair has previously been shown to be Ku-dependent NHEJ (43,44). In contrast, DNA with 3′ ddC was resected over time, giving rise to faster migrating products and only a small amount of supercoiled monomers. The intermediates still carried ^{32}P, consistent with the 5′→3′ direction of resection (45). There was also a small amount of linear dimers and multimers, most likely produced by resection-mediated end joining repair. DNA with 3′ biotin gave rise to more supercoiled monomer products than ddC DNA, but was mostly channeled to resection. The DNA with 3′ avidin was practically completely channeled to resection with no detectable supercoiled monomer products. Notably, avidin had no effect on the DNA with dC or ddC ends, suggesting that it was indeed the bulky adduct at the 3′ end that channeled the DNA away from NHEJ into the resection pathway.

Resection does not require the removal of the 3′ avidin

A bulky adduct at the 3′ end is expected to cause steric hindrance to the access by resection proteins. An important mechanistic question is if the adduct has to be removed before resection can start. The resection intermediates still carried ^{32}P, but it was three nucleotides inside the biotin-avidin end, so a cleavage might have occurred between biotin-avidin and the ^{32}P nucleotide. To address this question, we examined if the resection intermediates still retained biotin at the 3′ end. DNA with 3′ avidin was incubated in extracts and the intermediates at various times were isolated by a procedure that destroyed avidin (if still present). The presence of biotin at the 3′ end was then detected by resistance of the DNA to *E. coli* ExoI, a 3′ strand-specific ss-DNA exonuclease. As shown in Figure 1C, this nuclease could attack 3′ ss-overhangs even if the 3′ end carried a biotin, but not if the 3′ end was pre-incubated with avidin. In contrast, 3′ ss-overhangs with ddC at the end were degraded by the enzyme equally well with or without avidin. When the resection intermediates were incubated with *E. coli* ExoI, they were all degraded, confirming that they carried 3′ ss-overhangs (Figure 1D). However, when they were pre-incubated with avidin, the intermediates all became resistant to *E. coli* ExoI, indicating that they still carried biotin at the 3′ end. Because of the extremely high affinity of avidin for biotin ($K_a \sim 10^{15}$ M^{-1} (46)), these data suggest that resection proceeds without the prior removal of the avidin from the 3′ end.

DNA with 5′ damaged nucleotides or bulky adducts is also channeled to resection

We next examined the fate of DNA with damaged 5′ ends. DNA substrates were prepared by PCR with a 5.7 kb plasmid template and 5′ primers carrying various 5′ modifications in the presence of ^{32}P-dATP. They were incubated in *Xenopus* egg extracts and samples taken at the indicated times were analyzed by agarose gel electrophoresis. As shown in Figure 2A, DNA with 5′ -OH, which could easily be phosphorylated in the extract, was efficiently repaired into supercoiled and relaxed monomers and some dimers and multimers. In contrast, for DNA with 5′ p-Tyr, which mimics degraded Top2 (47), there was a lot of degradation and only a small amount of supercoiled monomer products. The levels of dimer and multimer products were also reduced when compared to DNA with the 5′-OH ends. DNA with 5′ biotin (attached to the C6 position of dG via a 14-atom linker) was very similar to DNA with 5′ p-Tyr except that the amount of supercoiled monomer product was

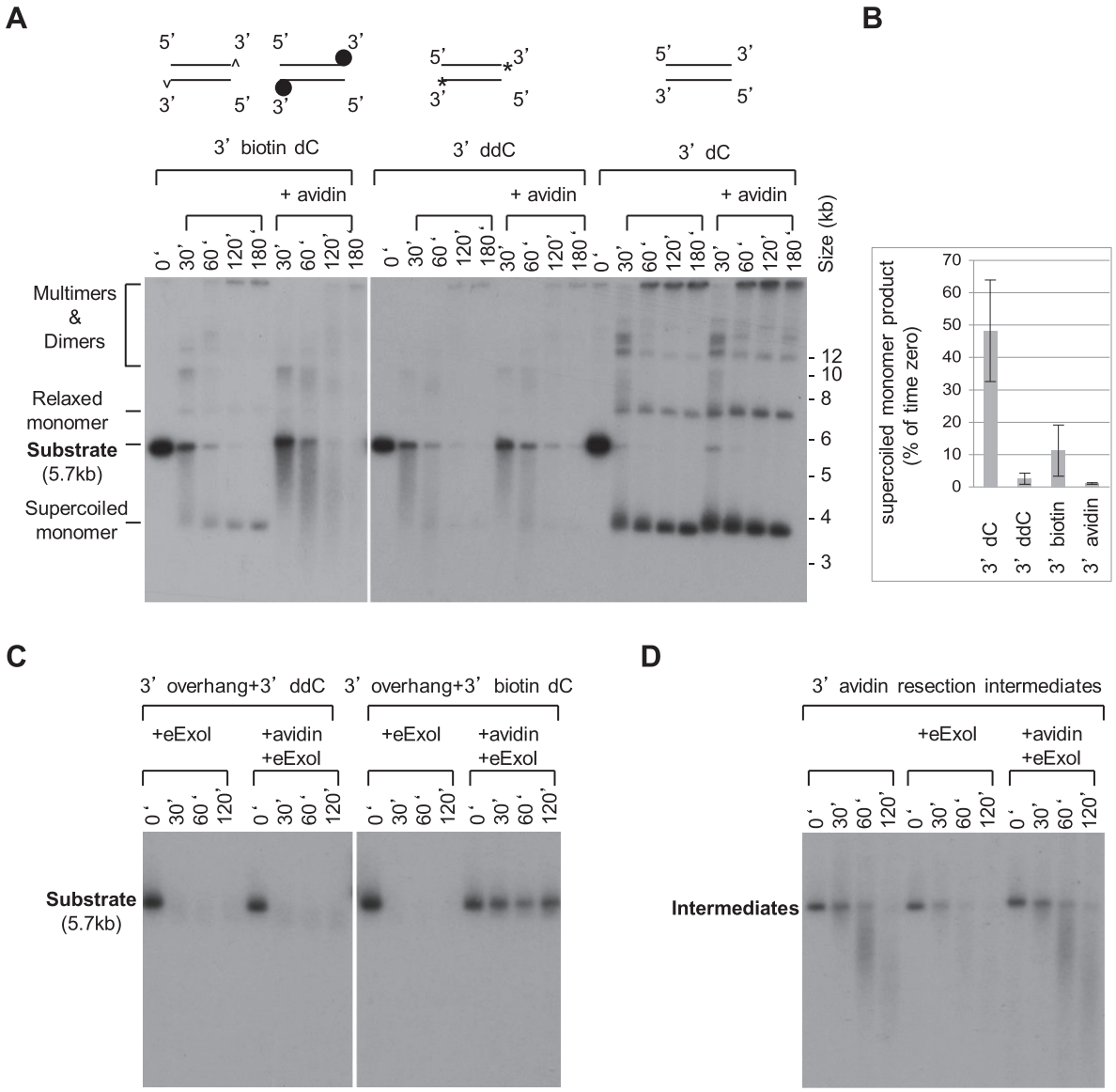

Figure 1. DNA with 3′ damaged nucleotides or bulky adducts is channeled to resection. (**A**) DNA substrates bearing different types of 3′ ends and labeled by ^{32}P at the third nucleotide from the 3′ end were incubated with *Xenopus* egg extracts for the indicated times. The products were analyzed on a 1% TAE-agarose gel. (**B**) Plot of the percentages of substrates converted into supercoiled monomer products at 180′. The averages and standard deviations were calculated with four sets of data. (**C**) Assay for detecting biotin at the 3′ end of ss-DNA. The ^{32}P-labeled 3′ ddC or biotin DNA with short 3′ ss-overhangs was pre-incubated with buffer or avidin and then treated with *E. coli* ExoI. The products were analyzed on a 1% TAE-agarose gel. (**D**) Avidin was not removed from the 3′ end of resection intermediates. 3′ avidin DNA was incubated in extracts for the indicated times, isolated, supplemented with buffer or avidin, and treated with *E. coli* ExoI. The products were analyzed on a 1% TAE-agarose gel.

much lower to a level barely detectable (P value = 0.001). 5′ biotin, unlike 5′ p-Tyr, cannot be cleaved off by tyrosyl-DNA phosphodiesterase 2 (TDP2) (48), so this observation suggests that the small amount of supercoiled monomer product with the 5′ p-Tyr substrate was most likely the result of TDP2 mediated NHEJ repair. For the DNA with 5′ avidin, which mimics intact Top2 covalently linked to the 5′ end, there was also efficient degradation and no detectable level of supercoiled monomer products but significant amount of dimers and multimers, mostly likely as the result of resection-mediated end joining. Collectively, these data demonstrate that DSBs with damaged nucleotides and bulky adducts at the 5′ end are channeled away from NHEJ to resection.

5′ bulky adducts are removed by resection

5′ bulky adducts pose an even more challenging steric hindrance than 3′ bulky adducts to the resection proteins. If resection still acts on the 5′ strand, then 5′ bulky adducts, unlike 3′ bulky adducts, have to be removed. In the above experiment, the DNA with 5′ avidin was uniformly labeled by ^{32}P, so it was not possible to know the direction of resection. To address this question, we purified the resection intermediates after 30 min of incubation in the extract. The

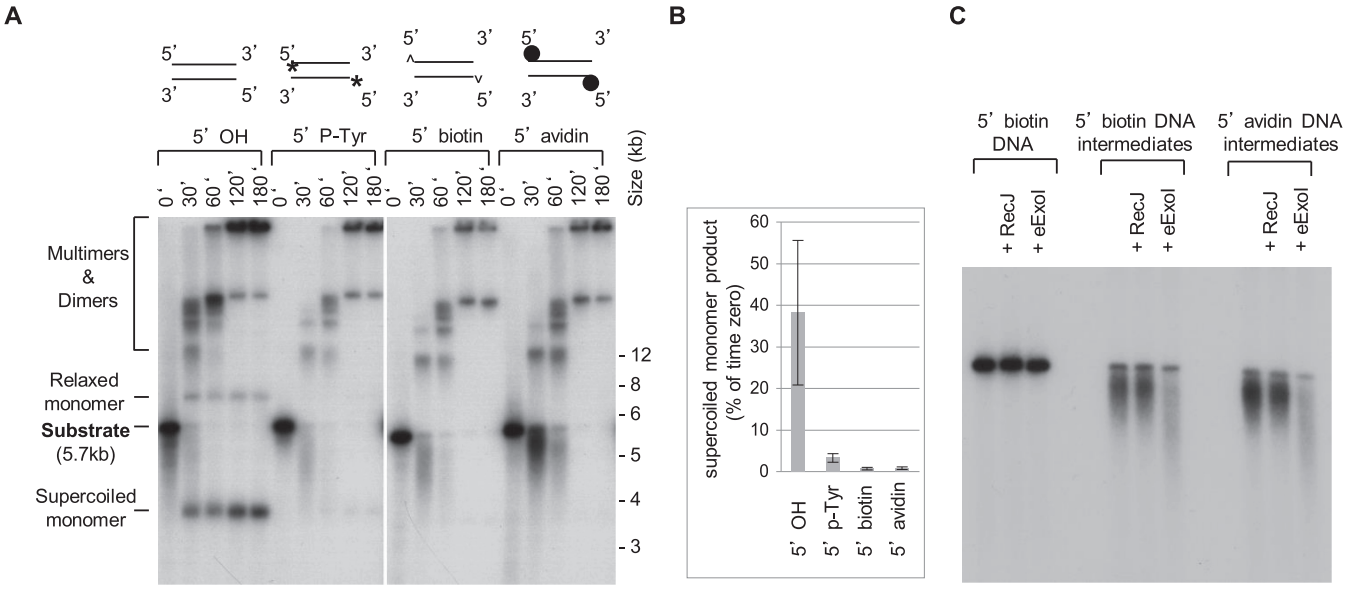

Figure 2. DNA with 5′ damaged nucleotides or bulky adducts is channeled to resection. (**A**) ^{32}P -labeled DNA substrates bearing different types of 5′ ends were incubated with *Xenopus* egg extracts for the indicated times. The products were analyzed on a 1% TAE-agarose gel and detected by exposing the dried gel to X-ray film. Avidin is bound to DNA ends via biotin. (**B**) Plot of the percentages of substrates converted into supercoiled monomer products at 180′. The averages and standard deviations were calculated with five sets of data. (**C**) Resection of 5′ avidin DNA proceeds in the 5′→3′ direction. 5′ avidin DNA was incubated with extracts for 30 min and re-isolated. They were incubated with buffer or avidin and then treated with *E. coli* ExoI or RecJ. The products were analyzed on a 1% TAE-agarose gel.

DNA was then subjected to treatment with two *E. coli* nucleases: ExoI, which degrades 3′ ss-DNA, and RecJ, which degrades 5′ ss-DNA. As shown in Figure 2C, treatment with *E. coli* ExoI, but not RecJ, made the intermediates migrate faster, indicating that they carried 3′ ss-overhangs, which is consistent with the 5′→3′ direction of resection. Therefore, the 5′ adduct, unlike the 3′ bulky adduct, is removed by the resection machineries.

Mre11 but not its nuclease activity is essential for the resection of DNA with 3′ damaged nucleotides or bulky adducts

While Mre11 is an important player in the initiation of resection, inactivating MRX in yeast only causes a 2-fold decrease in resection in proliferating cells (49). In addition, it has been shown to be essential for resection during G2 phase but not during S phase (50,51). One explanation for these observations is that Mre11 is only required for resection of some types of ends. Indeed we have previously shown that, while the resection of DNA with 3′ ddC requires MRN, the resection of DNA with 3′ ss-overhangs is independent of MRN (38). We thus tested if the substrates with different 3′ and 5′ damaged ends in this study might have different dependencies on Mre11 for resection. To do this, Mre11 was depleted from extracts with specific antibodies as previously described (38). The substrates carrying 3′ ddC or avidin were then incubated in Mre11 depleted or mock depleted extracts. The resection of both types of DNA was strongly inhibited by the depletion of Mre11 (Figure 3A and B). The inhibitory effect of Mre11 depletion was specific because resection was all efficiently complemented by the addition of the purified recombinant *Xenopus* MRN (Figure 3A and B). These data suggest that Mre11 is essential for the resection of DNA with damaged nucleotides or bulky adducts at the 3′ end.

We then examined if the nuclease activity of Mre11 is important for the resection of 3′ damaged ends. To do this, we expressed and purified a mutant MRN complex with the catalytic histidine of Mre11 mutated (Figure 3C). The corresponding mutation in *p. furiosus* Mre11 (H85) caused a complete inactivation of the nuclease activity (28). As expected, the mutant *Xenopus* MRN lost nuclease activity when assayed with M13 ss-DNA (Figure 3D). To assess whether the mutant MRN could support resection, the 3′ ddC or avidin DNA was incubated in Mre11-depleted extracts supplemented with equal amounts of either the wild-type or the mutant MRN complex. As shown in Figure 3A&B, the mutant MRN was less active than the wild-type in supporting the resection of DNA with 3′ ddC, but the majority of DNA was resected after three hours of incubation. Against the 3′ avidin DNA, the mutant was only slightly less active than the wild-type at the earlier time points, but caught up with the wild-type later. All the 3′ avidin DNA was degraded after two hours of incubation with either the wild-type or the mutant MRN. These data suggest that the nuclease activity of Mre11, while having some effect, is not essential for the resection of DNA with damaged 3′ ends, especially 3′ bulky adducts.

The Mre11 nuclease activity is critical for the resection of DNA with 5′ bulky adducts

We next determined if Mre11 and its nuclease activity are required for the resection of DNA with 5′ p-Tyr or avidin. The two DNA substrates were incubated in mock or Mre11-depleted extracts. As shown in Figure 4A&B, Mre11 depletion inhibited the resection of both DNA, especially that

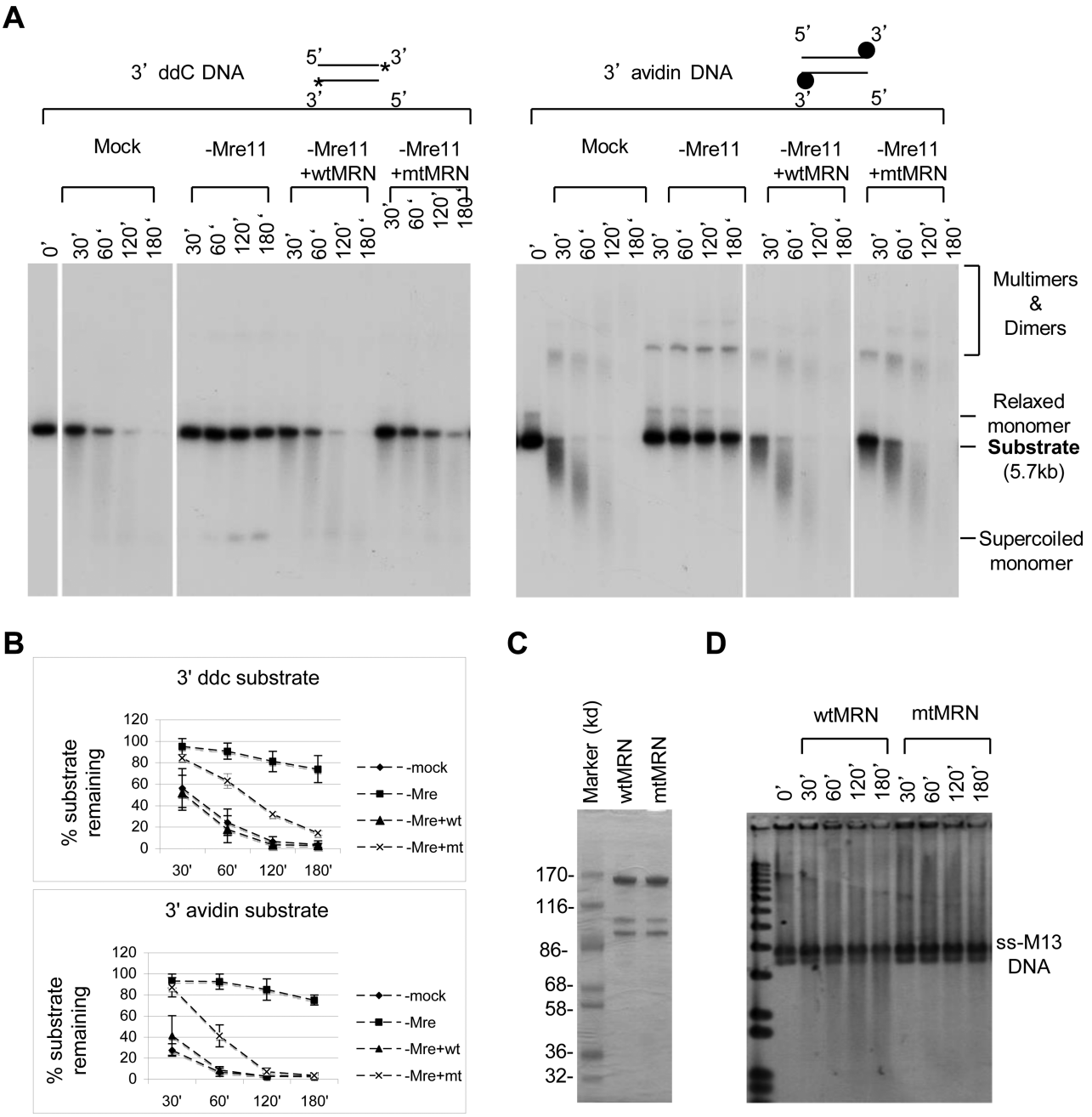

Figure 3. Mre11 but not its nuclease activity is essential for the resection of DNA with 3′ damaged nucleotides or bulky adducts. **(A)** Effect of MRE11 depletion on the resection of 3′ ddC and 3′ avidin DNA. The substrates were incubated in mock-depleted or Mre11-depleted extracts (with or without 8 ng/μl wild-type (wt) and mutant (mt) MRN) and the products were analyzed on a 1% TAE-agarose gel. (**B**) Plots of the amounts of 3′ ^{32}P on the remaining substrates at the indicated times. The averages and standard deviations were calculated with three sets of data. (**C**) A Coomassie blue stained SDS-PAGE gel showing the purified MRN complexes. (**D**) Ss-DNA endonuclease assay of the MRN complexes. Wild-type and mutant MRN (8ng/μl) were incubated with ss-M13 DNA (10 ng/μl) at 22°C for 30 min. The products were separated on a TAE-agarose gel and detected by SYBR Gold staining.

of 5′ avidin. More of the 5′ p-Tyr DNA was repaired, with the percentage of supercoiled monomer products increased by 3.6 (±1.2) fold in Mre11-depleted extracts. This suggests that the lack of resection provided more time for TDP2 to process the end for NHEJ. The 5′ avidin DNA, in contrast, was very stable, suggesting that the NHEJ factors were incapable of processing 5′ bulky adducts even in the absence of resection. The inhibition was reversed by the addition of the wild-type MRN, indicating that the effect was specific. The mutant MRN was quite effective at rescuing the resection of the 5′ p-Tyr DNA, only slightly less than the wild-type (Figure 4A&B). However, it was very inefficient against DNA with 5′ avidin. Even after 3 hours of incubation, there was still over 60% of the substrate left.

To determine if Mre11 and its nuclease activity were required for the removal of 5′ avidin, the DNA was incubated

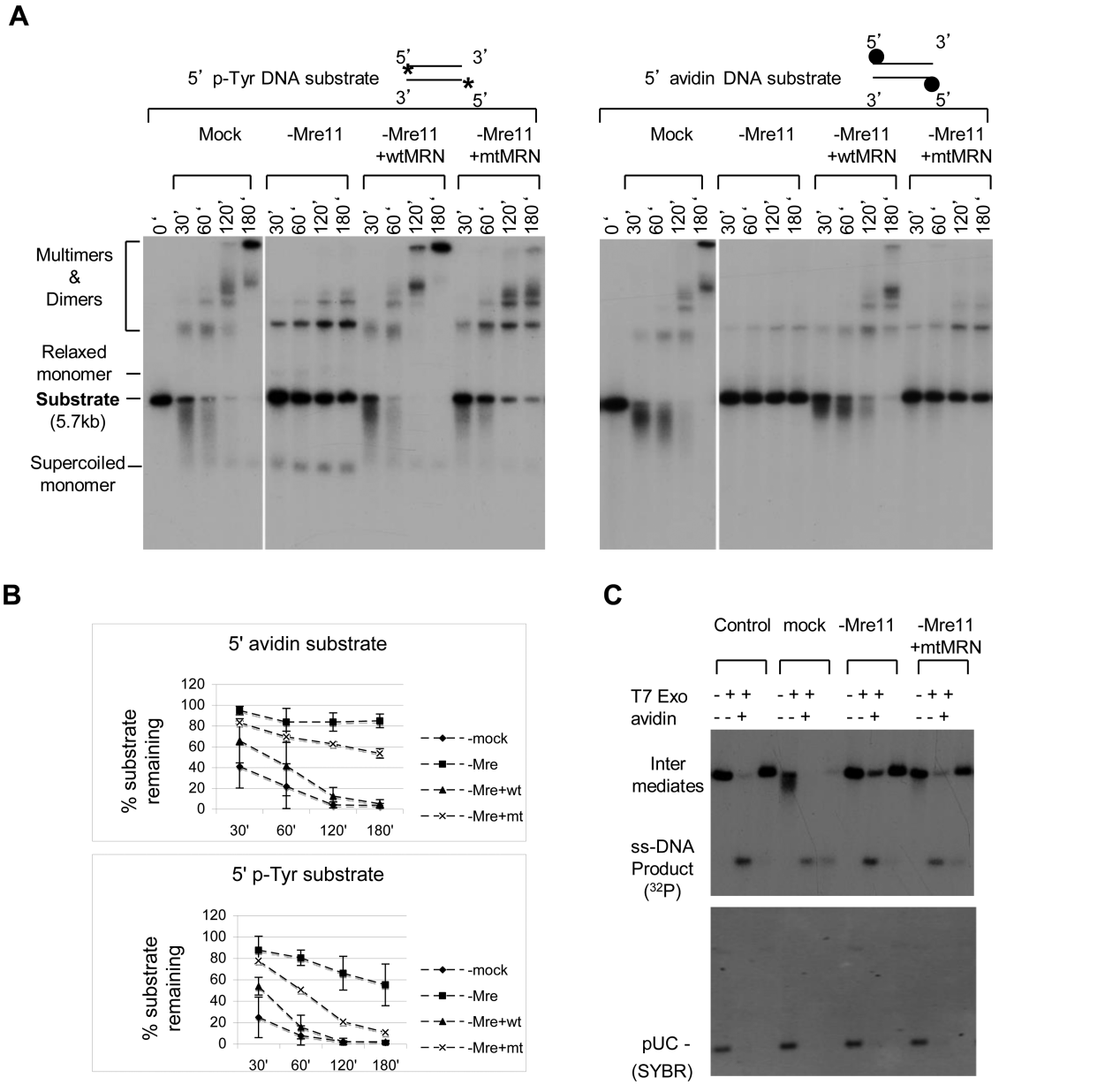

Figure 4. The Mre11 nuclease activity is critical for the resection of DNA with 5′ bulky adducts. **(A)** Effect of Mre11 depletion on the resection of 5′ p-Tyr and 5′ avidin DNA. The substrates were incubated in mock-depleted or Mre11-depleted extracts and the products were analyzed on a 1% TAE-agarose gel. (**B**) Plots of the amounts of ^{32}P on the remaining substrates at the indicated times. (**C**) Detection of 5′ biotin on the resection intermediates of 5′ avidin DNA. Control: untreated substrate. Mock and –Mre11: intermediates isolated after 30 min in the indicated extracts. They were pre-incubated with buffer or avidin, and then treated with T7 Exo. (T7 Exo falls off DNA once the two enzyme molecules meet in the middle, resulting in the accumulation of ss-DNA of the 3′ half). The reactions also contained a plasmid (pUC) to serve as a control for digestion. The products were analyzed on a 1% TAE-agarose gel, stained with SYBR Gold, and dried for exposure to X-ray film.

in mock or Mre11-depleted extracts for 30 min and then re-isolated by a procedure that destroys avidin (if still present). The existence of 5′ biotin was detected by using the bacterial T7 Exo, a 5′→3′ ds-DNA exonuclease. T7 Exo could efficiently degrade ds-DNA with a biotin at the 5′ end (Figure 4C). However, if the DNA was pre-incubated with avidin, it became resistant to T7 Exo. Notably, linear pUC DNA (with no biotin at the end) in the same reactions was still sensitive to T7 Exo, indicating that it's the avidin at the 5′ end that blocked T7 Exo. The DNA isolated from the mock-depleted extracts was sensitive to T7 Exo, even after pre-incubation with avidin (Figure 4C). This is consistent with partial resection that had removed the 5′ avidin. In contrast, the DNA isolated from Mre11-depleted extracts, supplemented with either buffer or the mutant MRN complex, was sensitive to T7 Exo, but became resistant after pre-incubation with avidin. This indicates that the biotin (and thus avidin) at the 5′ end was stable in these extracts. Together these data suggest that Mre11 and its nuclease activity are critical for the removal of 5′ bulky adducts.

The structure of ends also determines the requirement for CtIP

The data presented above suggest that the requirement for the Mre11 nuclease activity in resection is determined by the structure of ends. The Mre11 nuclease activity is necessary only for the DNA with 5′ bulky adducts, but dispensable for other types of DNA, in particular the type with 3′ bulky adducts. However, the Mre11 endonuclease activity is activated by CtIP on DNA with bulky adducts regardless of their placement at the 5′ or the 3′ end of DNA (34). An important mechanistic question is what role CtIP, the other key factor for the initiation of resection, plays in the resection of these types of DNA. To address this question, CtIP was depleted from *Xenopus* egg extracts as previously described (38). The effect on resection of DNA with 3′ avidin or 5′ avidin DNA was then analyzed. As shown in Figure 5A&C, CtIP depletion strongly inhibited the resection of the 3′ avidin DNA. However, the defect could be efficiently complemented by the addition of 2x excess MRN. These effects are similar to those for the 3′ ddC DNA and are not due to spurious co-depletion of MRN (38). The mutant MRN exhibited comparable activity to the wild-type MRN in complementing CtIP-depleted extracts in supporting the resection of 3′ avidin (Figure 5A&C). In contrast, CtIP depletion also strongly inhibited the resection of 5′ avidin DNA, but this inhibition could not be rescued by excess MRN (Figure 5B&C). Analysis of the intermediates by T7 Exo showed that the 5′ biotin was still intact in the absence of CtIP, and this defect could not be overcome by the addition of excess MRN (Figure 5D). Together, these data suggest that the requirement of CtIP for resection is also determined by the structure of ends. It is absolutely necessary for DNA with 5′ bulky adducts but dispensable for DNA with 3′ bulky adducts.

MRN can directly stimulate resection by an Mre11 nuclease-independent mechanism

The observation that the Mre11 nuclease activity is essential only for the resection of DNA with 5′ bulky adducts suggest that MRN can promote resection not only by a nuclease-dependent mechanism but also by a nuclease-independent mechanism, especially on ends with 3′ bulky adducts. However, it is also possible that in the absence of Mre11 nuclease activity, another nuclease in the extract, such as CtIP, might provide a substitution. To rigorously test the hypothesis of a nuclease-independent mechanism for Mre11, we compared the activity of the wild-type MRN complex and the mutant MRN complex in stimulating the resection activity of the purified Exo1 enzyme on DNA with 3′ bulky adducts. Exo1 has intrinsic 5′→3′ ds-DNA exonuclease activity, even against DNA with 3′ biotin (Figure 6A). This activity was inhibited by the presence of avidin at the 3′ end. Similarly, 3′ avidin also inhibited the activity of lambda exonuclease, another enzyme that degrades ds-DNA in the 5′→3′ direction. These observations confirm that a 3′ bulky adduct poses a general steric hindrance to DNA exonucleases. However, the steric hindrance to Exo1 could be completely overcome by the wild-type MRN complex. In contrast, MRN showed much weaker relief to the steric hindrance to lambda exonuclease. These results demonstrated that the stimulatory effect of Exo1 by MRN is specific, consistent with the known protein-protein interaction between the two (38,40,52). When the nuclease mutant MRN was examined, it displayed an equal activity in stimulating Exo1's degradation of the 3′ avidin DNA (Figure 6B). Together, these data demonstrate that MRN can indeed stimulate resection by a mechanism independent of the Mre11 nuclease activity.

DISCUSSION

In this study, we investigated the effect of end structure on the choice of DSB repair pathways and mechanism of resection. Our major findings are: (i) DNA with normal nucleotides at ends are channeled to NHEJ; (ii) DNA with damaged nucleotides or bulky adducts at ends are refractory to NHEJ and preferentially channeled to 5′ resection; (iii) the resection of DNA with 3′ bulky adducts does not require the removal of adducts; (iv) in contrast, the resection of DNA with 5′ bulky adducts requires the removal of adducts; (v) the MRN complex is absolutely required for the resection of all the types of DNA tested; (vi) however, the nuclease activity of Mre11 is required only for the resection of DNA with 5′ bulky adducts; (vii) CtIP is also required only for the resection of DNA with 5′ bulky adducts; (viii) MRN can promote resection by a nuclease-dependent mechanism as well as a nuclease-independent mechanism. Together, these findings, when combined with other studies in literature, have elucidated the general principles of how end structures impact the choice of repair pathways and the dependence on Mre11 nuclease activity.

The structure of ends has three types of effect on the choice of DSB repair pathways. DSBs with normal ends such as those generated by site-specific endonucleases are ideal substrates for both the NHEJ and the HDR repair pathways. In budding yeast, the dominant pathway is HR, but in higher eukaryotes, NHEJ is the dominant pathway, even during S and G2 phases, most likely due to the abundance of Ku (53). Our data provide direct biochemical evidence that normal ends are indeed preferentially repaired by NHEJ in higher eukaryotes. Compared to DSBs with normal ends, DSBs with simple damaged nucleotides are more difficult to repair by NHEJ. Our data show that these DSBs are efficiently channeled to resection for HDR. However, NHEJ can still occur, especially if resection is blocked, as illustrated by the DNA with 5′ p-Tyr. These observations are in agreement with *in vivo* studies showing that 15–20% of ionizing radiation induced DSBs in human G2 cells are channeled to resection, but if resection is inhibited, they can still be repaired by NHEJ (41). Structurally, these ends can be recognized by Ku, or by repair enzymes, such as Tdp2, that can remove the damage. NHEJ and HDR are thus still effectively competing, but the equilibrium favors HDR. In sharp contrast, DSBs with bulky adducts at ends can only be channeled to resection. If resection is blocked by depletion of Mre11 or CtIP, ends still cannot by repaired by NHEJ. Structurally, these types of ends are recognized by MRN but not by Ku (34,54). This provides a mechanistic explanation for the exclusive channeling of them to resection. *In vivo*, Spo11-linked DSBs are indeed channeled to resection in yeast meiotic cells. In *Xenopus* egg extracts and

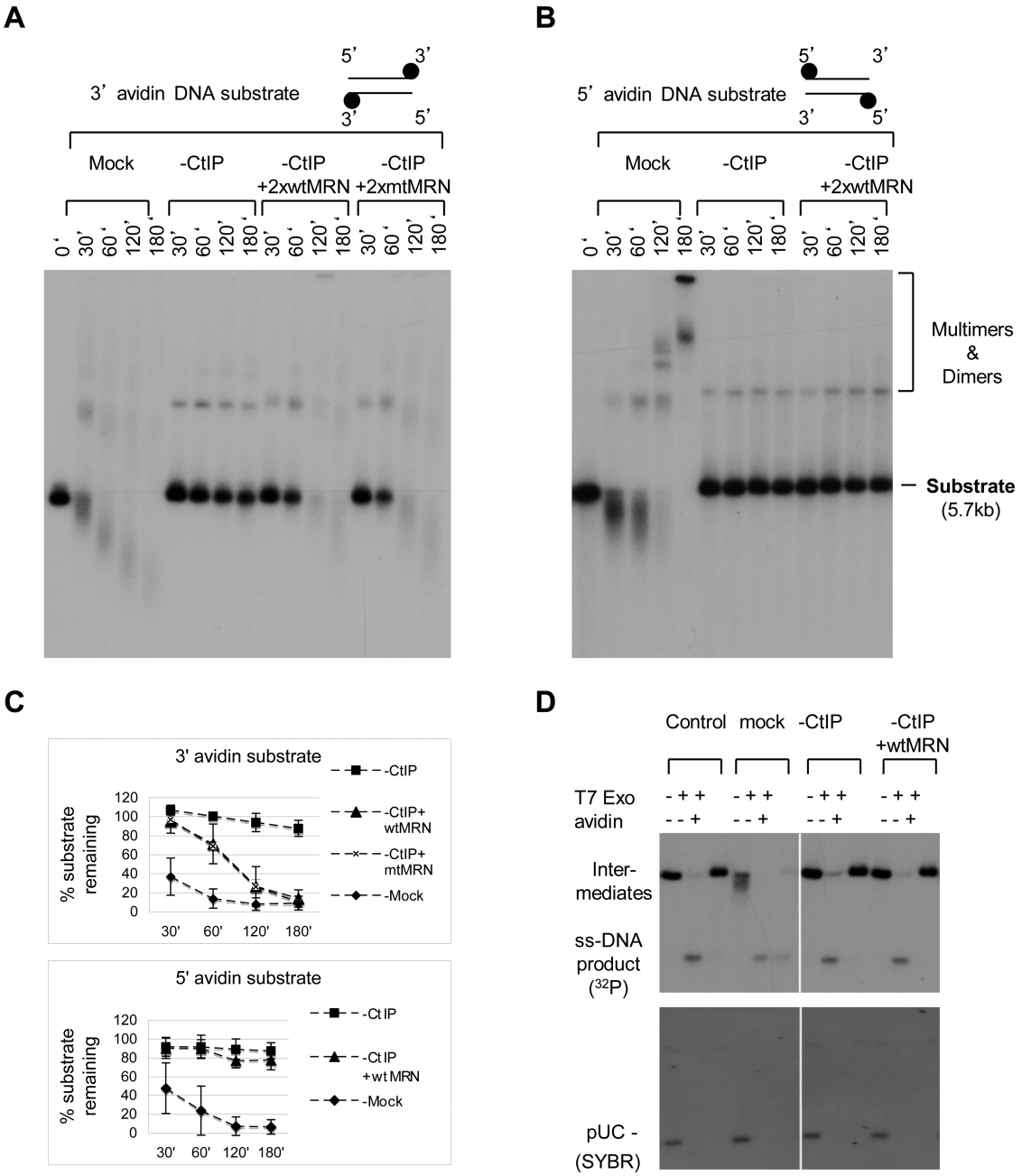

Figure 5. The structure of ends affects the dependence on CtIP for resection. (**A**) Effect of CtIP depletion on 3′ avidin DNA resection. DNA was incubated in mock-depleted or CtIP-depleted extracts supplemented with 2x excess wild-type or mutant MRN (16ng/μl final concentration). (**B**) Effect of CtIP depletion on 5′ avidin DNA resection. DNA was incubated in mock-depleted or CtIP-depleted extracts supplemented with 2× excess wild-type MRN (16ng/μl final concentration). (**C**) Plots of the amounts of 3′ ^{32}P on the remaining substrates at the indicated times. (**D**) Detection of 5′ biotin on the resection intermediates of 5′ avidin DNA. The intermediates were isolated after 30 minutes incubation and analyzed as in Figure 4.

mitotic cells, Top2-linked DSBs generated during S phase are also efficiently resected (55–57).

Despite the diversity of end structures, all the DNA substrates tested in this study absolutely require Mre11 for resection. *In vivo*, most of the studies suggest that Mre11 is important for the resection of 'clean ends' as well as 'dirty ends'. However, the nature of 'dirty ends', usually induced by ionizing radiation, laser, or drugs, is ill-defined and most likely represents a mixture with various types of damaged nucleotides and/or bulky adducts at either 5′ or 3′. Our study provides a more definitive answer that MRN is indeed required for resection regardless of end structure. The only exception is the DNA with a long 3′ overhang, which is channeled to MRN-independent resection (38). Structurally, this type of DNA is a poor substrate for the binding of Ku but ideal for the entry of RecQ-type helicases (24,58–60). *In vivo*, there are also exceptions. In budding yeast, MRX is essential for resection during G2 phase but not during S phase (50,51). In human cells Mre11 does not affect the resection of ends of reversed replication forks (61).

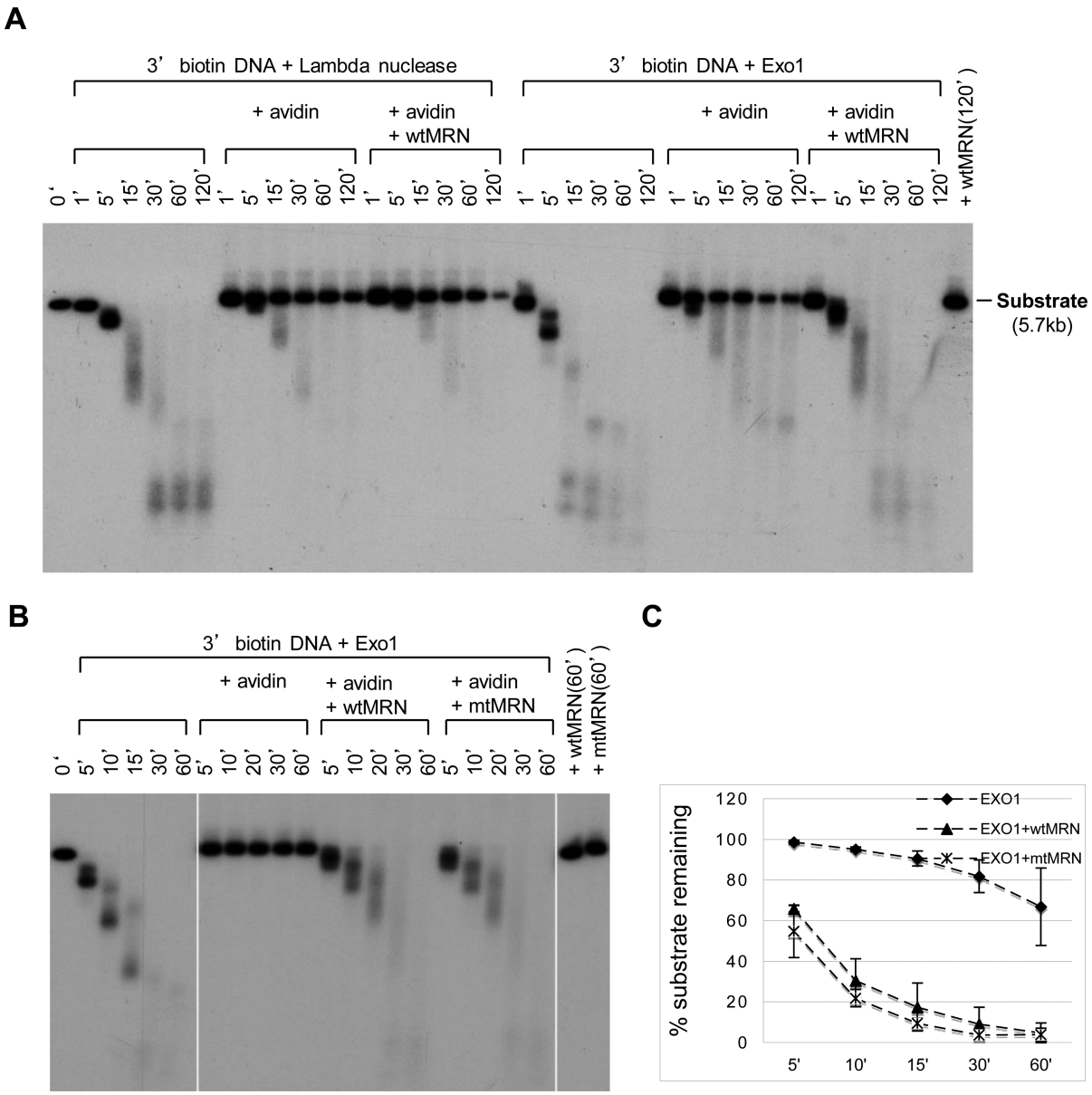

Figure 6. MRN can stimulate resection by an Mre11 nuclease-independent mechanism. (**A**) Effect of MRN on the activities of lambda exonuclease and Exo1 against 3′ avidin DNA. (**B**) Comparison of the wild-type and mutant MRN complexes on the stimulation of Exo1 activity against 3′ avidin DNA. (**C**) Plot of the amounts of 3′ ^{32}P on the remaining substrates at the indicated times. The averages and standard deviations were calculated with three sets of data.

A reasonable hypothesis is that these ends are converted to 3′ ss-overhangs after the collapse or reversal of replication forks due to the nature of lagging strand synthesis. Further research is required to test this hypothesis.

The nuclease activity of Mre11 has long been proposed to be critical for the resection of 'dirty ends' (35,62–65). Our data partially confirm this hypothesis but also suggest that not all 'dirty' ends are equal. 5′ bulky adducts absolutely require the Mre11 nuclease activity and CtIP. This is consistent with the hypothesis that the mechanistic role of Sae2 (and by extrapolation CtIP) in the removal of 5′ bulky adducts is to stimulate the endonuclease activity of Mre11 (34). However, due to technical difficulties with the preparation of active CtIP protein, we could not conduct rescue experiments to compare the wild-type and the putative nuclease-dead CtIP proteins. As such, our data cannot rigorously rule out the possibility that CtIP might also function as a nuclease (66,67). *In vivo*, resection of *S. cerevisiae* meiotic DSBs, which carry Spo11 at the 5′ end, is dependent on the Mre11 nuclease activity and Sae2 (30,35,68). However, the Mre11 nuclease activity is not absolutely required for the resection of DNA with other types of ends, in particular ends with 3′ bulky adducts. This is in agreement with studies in *S. cerevisiae* showing that the Mre11 nuclease mutant causes only a slight delay in the resection of IR-induced DSBs, most of which carry simple damaged bases at ends (69). Enzymatically, 3′ bulky adducts also induce Sae2 to activate Mre11's endonuclease activity to cleave the 5′ strand

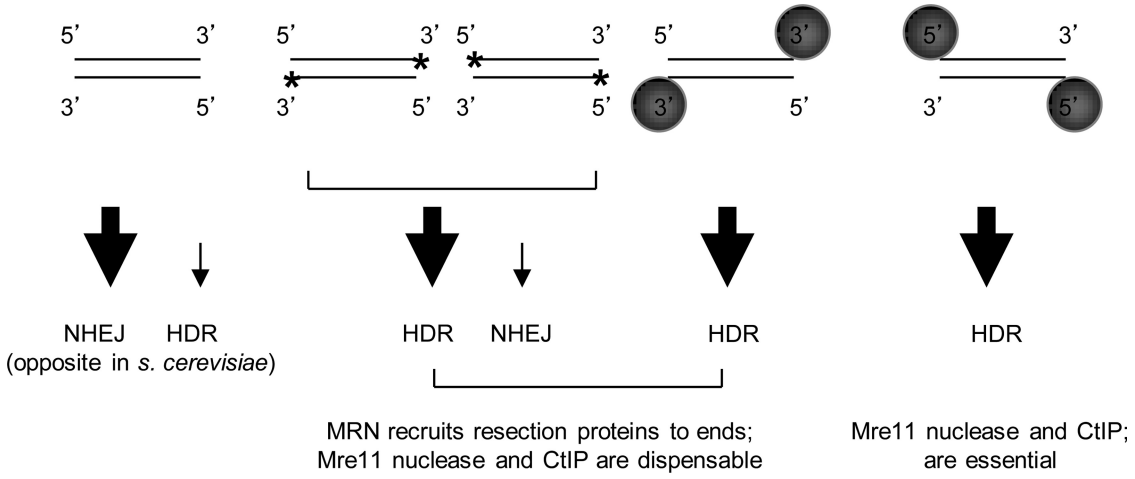

Figure 7. Model for the repair pathway choice and Mre11 nuclease dependence of different types of ends. Normal ends are predominantly channeled to resection (except in *S. cerevisiae*). Ends with minor damages are preferentially channeled to resection. Ends with bulky adducts are exclusively channeled to resection. Resection is absolutely dependent on Mre11, but its nuclease activity and CtIP are essential only for ends with 5′ bulky adducts.

(34). However, our data suggest that this cleavage, if it does happen in total extracts or cells, is not essential for MRN to initiate resection. Structurally, 3′ bulky adducts pose a general steric hindrance to both Exo1 and lambda exonuclease, but MRN can efficiently help Exo1 overcome this hindrance and attack the 5′ end, which is still accessible. The nuclease mutant MRN is fully capable of stimulating the purified Exo1 on ends with 3′ avidin. It has been previously shown that the budding yeast Mre11 nuclease mutant can stimulate Exo1 to resect DNA, but only in the presence of Sae2, which also has nuclease activity (40). Our data thus provide more definitive evidence that MRN can directly stimulate resection by a nuclease-independent mechanism. In support of this mechanism, MRN is known to interact with resection proteins to promote their recruitment to DNA ends (38). In the absence of Mre11 nuclease activity, the actual degradation of 5′ strand DNA is most likely carried out completely by Exo1 and DNA2 as suggested by previous studies (45,70). It should be emphasized that the 3′ adduct eventually has to be removed for the repair to be completed. The enzyme(s) responsible for this post-resection step is currently unknown. One candidate is still the Mre11 nuclease activity, which has been shown to facilitate the removal of Topoisomerase 1 (Top1) covalently trapped to the 3′ end of DNA by camptothecin in the fission yeast *S. pombe* (71). If so, the reaction mechanism must be distinct from that of 5′ bulky adduct removal because it is stimulated rather than inhibited in the absence of Ctp1, the *S. pombe* homolog of Sae2/CtIP (71).

In summary, our study suggests a model for the pathway choice of DSB repair and the role of Mre11 nuclease activity in resection (Figure 7). Normal ends are efficiently repaired by NHEJ or HDR and the relative contribution of each pathway is dependent on organism, cell type, and cell cycle stage. In contrast, 'dirty' ends are more difficult to repair by NHEJ and consequently channeled to resection for HDR. However, if resection is inhibited, such as during G1 phase, structurally simple 'dirty' ends can still be channeled to NHEJ. In contrast, 'dirty' ends with bulky terminal adducts have no choice but resection. Resection of all ends except those with 3′ overhangs is dependent on MRN. MRN can initiate resection by two mechanisms. One is nuclease-dependent by introducing a cut near the 5′ end and other is nuclease-independent by directly recruiting resection proteins to ends.

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